Physicochemical factors affecting the rapid bactericidal efficacy of the phenolic antibacterial Triclosan*

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ABSTRACT

The antimicrobial activity of triclosan (2,4,4'-trichloro-2'-hydroxydiphenyl ether, TCS) in aqueous solutions is shown to directly depend upon two key physicochemical parameters: percent saturation and saturation solubility. Saturated solutions of TCS in water, in water-propylene glycol mixtures, and in aqueous surfactant systems are shown to effect rapid, potent bacterial reductions (e.g., >4log$_{10}$ reduction of *Staphylococcus aureus* in 15 seconds contact time in a time kill suspension test). In surfactant solutions, increasing the surfactant:TCS ratio causes a decrease in antibacterial efficacy, consistent with a model for micellar solubilization where the micelle binding constant, $K(=\frac{X}{c_w})$, increases with decreasing TCS concentration in the micelles (X), resulting in decreased concentration of bioavailable TCS in the water (continuous) phase (c_w). The rapid and potent reductions

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of bacteria reported here support the existence of a non-specific mode of action for TCS, such as gross membrane disruption, in addition to the specific modes of action reported by others.

KEYWORDS

triclosan; antimicrobial activity; antimicrobial resistance

INTRODUCTION

Triclosan (2,4,4'-trichloro-2'-hydroxydiphenyl ether, TCS) has been used for more than 30 years in antibacterial personal care products including skin cleansers and oral care products. Products formulated with TCS have exhibited a broad spectrum of antibacterial efficacy as measured in standard tests. This paper explores the physicochemical factors governing the efficacy of phenolic antibacterials in general and describes formulation approaches to maximize the effectiveness of TCS.

Due to their sparing water solubility, phenolic biocides are typically formulated with surfactants to effect stable formulations. In fact, surfactants are excellent solubilizers for phenolic biocides, increasing the solubility of triclosan, for example, several hundred-fold over its solubility in water. However, such a high level of solubilization implies that the biocide is highly partitioned to the micellar pseudophase[1]. This strong partitioning effect, and its impact on phenolic biocide efficacy, has been reported in the literature for many years[2] and was explained from a physicochemical perspective by Allawala and Reigelman[3,4]. Allawala and Reigelman showed that a governing factor of efficacy of a phenolic agent in surfactant solution was the percent saturation of the phenolic (which is

an index of its thermodynamic activity) in the solution, and not simply its total concentration; the two can be very different depending on the mode of solubilization. Mitchell[5] extended this approach to chloroxylenol, a phenolic biocide. Mitchell showed that a saturated solution of chloroxylenol in water had the same bactericidal activity as saturated surfactant solutions of chloroxylenol containing up to 100-fold greater amounts of the biocide. Kostenbauder[6] has summarized the early work on phenolic biocide interaction with surfactants. To paraphrase, the efficacy of an antimicrobial agent is correlated to the rate at which it gains access to the biophase on the site at which it acts. The driving force that determines this rate of transport is the difference in chemical potential of the biocide between the site at which it acts and the external aqueous phase. We can make use of the relative thermodynamic activity in place of the absolute chemical potential, given a proper choice of reference states for the solution components. The thermodynamic activity of the solute (biocide) can be conveniently indexed by its percent saturation in solution given an appropriate choice of reference state (i.e., saturated solution reference state for the solute). Surfactants are capable of greatly reducing the thermodynamic activity, and hence percent saturation, of a biocide in solution without affecting the total concentration of the biocide at all. We explore the profound effect this has on triclosan efficacy in this paper.

Time-dependence, or kinetics, is also an important aspect of antimicrobial activity. One factor influencing the kinetics of antimicrobial activity of triclosan is the saturation solubility of the biocide. Effects of saturation solubility on kinetics are most apparent in saturated solutions of the biocide having relatively low saturation solubility; we have made saturated solutions with different concentrations of TCS by the use of co-solvency

with propylene glycol and water mixtures. We shall see how the influence of the saturation solubility on bacterial kill kinetics provides useful insights into maximizing biocide efficacy.

To date, the open literature has lacked a complete exploration of the factors governing TCS efficacy that we address here. Attempts to overcome the effects of surfactant solubilization, or more properly "over-solubilization", of TCS have been reported[7-9]. However, these earlier attempts have been limited to narrow pH ranges or specific pH values (e.g., <4 or >9), which may limit applicability. This so-called "de-blocking" technology (see reference 7) has never received a full exposition in the open literature. We have disclosed highly efficacious formulations with triclosan[10-12], which operate according to the principles disclosed here, without the pH limitations of the earlier work.

MATERIALS AND METHODS

Materials

Triclosan was obtained from Ciba Specialty Chemicals (trade name Irgasan DP300); it was assayed as >99% pure. Propolyene glycol (PG) (USP grade) was obtained from Dow Chemical and was >99% pure. Sodium lauryl sulfate was obtained as sodium dodecyl sulfate from BDH Biochemicals and was 99% pure. Ammonium lauryl sulfate was obtained as a 28.3% aqueous solution (trade name Standapol A) from Henkel Corp. and was used as received. Water was deionized.

Methods

Preparation of saturated solutions. Saturated solutions were prepared in one of several ways. For solutions without surfactant or solvent, excess TCS was stirred in water of 40-

45 °C for at least 8 hours to form an aqueous dispersion. The dispersion was then filtered and cooled to 20 °C. Typically, fine needle crystals would form in the filtrate solution indicating a saturated solution. In most cases, these crystals were filtered from the solution prior to testing. Appropriate dilutions of this concentrated stock solution were made to produce samples with saturation levels less than 100%. For samples with PG, TCS was first dissolved in the solvent and diluted to a final target concentration with water. A seed crystal of TCS was then added to the solution and the solution was allowed to stand at about 20 °C to crystallize. Precipitation was assessed using a bright, focused beam of visible light from a hand held flashlight. For a given concentration of PG, the maximum additive concentration of TCS was determined by successively halving the concentration of TCS until the limit of TCS solubility was reached. For solutions containing ammonium lauryl sulfate (ALS), the TCS was dissolved in the concentrated ALS aqueous solution by mixing and warming the solution to 35 – 45 °C until the TCS dissolved (about 1 hour); the maximum additive concentration was determined as in the PG samples, described above. For solutions containing sodium lauryl sulfate (SLS), the SLS was first dissolved in water, and then the TCS was added. The maximum additive concentration was then determined from the precipitation/non-precipitation behavior as described above. For a thorough exposition of maximum additive concentration determination as performed here, the reader is referred to the text by Elworthy, et. al[13]. TCS concentrations were determined by HPLC.

Time Kill Method. Bactericidal activity was assessed by a time kill (suspension) test[14]. The method included procedures to confirm neutralization of TCS under the test conditions to ensure accurate measurement of cidal action vs. inhibition. The following

bacteria were used for testing: *Staphylococcus aureus, ATCC# 6538; Escherichia coli, ATCC# 11229; Klebsiella pneumoniae, ATCC# 10031.* The inocula were prepared by growing the appropriate bacterial culture on agar. The bacteria were then harvested from the surface of the agar with sterile physiological saline and the population was adjusted to approximately 1.0 x 10^8 colony forming units per ml (cfu/ml). To conduct the test, a beaker containing 100.0 ml of the test solution was inoculated with 1.0 ml of the appropriate bacterial suspension (while maintaining constant stirring). At the predetermined contact time (e.g., 30 seconds), 0.1 ml of the inoculated test solution was transferred into 9.9 ml of Dey and Engley Neutralizing Broth (D/E). Serial ten-fold dilutions were performed using Butterfield's Phosphate Buffer with Polysorbate 80, and the surviving bacteria were enumerated using Trypticase Soy Agar with Lecithin and Polysorbate 80 (BBL). The plates were incubated at 35° C for 48 hours. Standard plate counting procedures were used to count colony forming units. Bacteria counts recovered were converted into $\log_{10}$ counts. The control count (numbers control) was determined by conducting the procedure as described with the exception that 100 ml of sterile de-ionized water is used in place of a test solution. Each time point was tested in triplicate. The $\log_{10}$ reduction was the difference between the $\log_{10}$ of the numbers control cfu/ml and the $\log_{10}$ of the test sample survivors cfu/ml.

RESULTS AND DISCUSSION

Triclosan in Water – Effect of Saturation

We prepared saturated and unsaturated solutions of TCS in water to investigate its inherent antimicrobial activity as well as the effect of saturation state on efficacy. Figure 1 shows a plot of the $\log_{10}$ reduction of three bacterial strains (*S. aureus, E. coli, and K.*

pneumoniae) in a time kill test at a contact time of 5 minutes at 25 °C versus percent saturation of TCS. The absolute concentrations of TCS (in ppm) are shown in brackets on the graph. Thus the saturated solution corresponds to ~10 ppm TCS in water at room temperature. A nearly linear relationship between percent saturation and $\log_{10}$ reduction is seen for each bacterial strain. While it is true that plotting total concentration on the independent axis would leave the plot essentially unchanged, this is only true because of the extremely dilute solution state when TCS is studied in water alone (i.e., nearly an ideal solution with activity coefficient close to unity over the composition range). Perhaps the most striking feature of this graph is the very high $\log_{10}$ reduction of bacteria achieved by the saturated TCS solution. Such a high $\log_{10}$ reduction at a relative short contact time of five minutes suggests a non-specific mode of action consistent with gross membrane disruption.

Triclosan in Water/Solvent Mixtures – Effect of Increasing Solubility on Bacterial Kill Kinetics

Propylene glycol (PG) is an excellent solvent for TCS and is water-miscible. We used PG as a co-solvent to prepare saturated solutions of TCS in PG/Water mixtures in various rations, each with an increasing solubility of TCS. We then tested these solutions for antimicrobial efficacy against *S. aureus* at different time points. Table 1 contains the results, indicating the TCS saturation solubility and the time for each solution to effect at least a 4 $\log_{10}$ reduction of the bacteria. We can very clearly see from the data in Table 1 that the saturation solubility of TCS has a strong effect on the kinetics of antimicrobial activity. Again, these results highlight the rapid cidal efficacy of TCS when present in solutions at a state of maximum thermodynamic activity and when present in a sufficient

amount. The ability to effect a $4\log_{10}$ reduction in 15 seconds gives further support to a non-specific mode of action for TCS under these conditions. Solutions of 50% PG in water alone gave no significant bacterial reductions within the time frame of these experiments.

Triclosan in Aqueous Surfactant Solutions – Effect of Micellar Solubilization

When TCS is solubilized in water with the aid of surfactants, we hypothesize that the TCS molecules are dissolved to a great extent in the surfactant micelles, based on the dramatic increase in TCS solubility when surfactant is present. Accordingly, TCS will be distributed between the micelles and the continuous aqueous phase of the solution according to some partition law. When the solution is saturated with TCS, both the micellar pseudophase and the bulk aqueous phase of the solution will be saturated, although the concentrations of TCS in each phase will necessarily be quite different. The saturation state of the solution can be manipulated in two ways. Either the total concentration of TCS can be changed, or the total surfactant concentration (and hence the concentration of micelles) can be changed. In both cases, the surfactant to TCS ratio is also changed, thus altering the saturation percentage of the micelles. We have studied the effect of TCS saturation on antibacterial efficacy in the presence of two surfactants, sodium lauryl sulfate (SLS) and ammonium lauryl sulfate (ALS).

Table 2 shows time kill results of solutions of TCS in aqueous ALS against *E. coli* at a contact time of 1 minute. For solutions with ALS concentration of 1.35%, the saturation percentage of TCS is manipulated from 100 to 50 by adjusting the total TCS concentration from 0.3% to 0.15%. The antimicrobial activity decreases from a $\log_{10}$ of 3.95 to 1.17 over this range of saturation. To emphasize that this effect is not merely

indicative of a dose response of TCS, we prepared a solution TCS at 100% saturation comprising a total TCS concentration of 0.15% and an ALS concentration of 0.67%. This solution gave a 3.63 $\log_{10}$ reduction (cf. Table 2), similar to the other saturated solution in the table containing twice as much TCS. Thus, we understand that as the surfactant to TCS ratio increases, progressively less TCS is in the continuous aqueous phase (i.e., bioavailable state); i.e., micellar partitioning increases at progressively lower solubilizate concentrations. In all the studies reported here, the pH of the solutions was near 7.

Rapid Cidal Activity and Mode of Action of Triclosan

We have provided substantial evidence that triclosan can effect rapid bacterial cell death when present in solution in a state of near maximum bioavailability (i.e., maximum thermodynamic activity) and when present in sufficient quanitity. This is in accord with data presented by Regos and Hitz[15] suggesting that, at concentrations greater than 10 ppm in water, triclosan induces a release of cytoplasmic material from bacterial cells by irreversible disruption of the cell membranes. Prompted by the work of McMurry et al.[16], reports have appeared in the literature postulating that the mode of action of triclosan is inhibition of the enoyl-acyl carrier protein reductase, sparking concerns about the potential emergence of possible triclosan-resistant bacteria. This concern has been prompted, in part, by the observation of laboratory strains of bacteria exhibiting genetically mediated TCS insusceptibility (as measured by elevated MIC values). As our work clearly shows, triclosan has multiple modes of action, including a non-specific mode involving gross membrane disruption resulting in rapid cell death. The potency and speed of triclosan efficacy at high saturation states cannot be attributed to modes of

action involving inhibition of enzymatic pathways, which would necessarily affect cellular metabolism or division, but not result in such catastrophic cell death in the time frames measured.

Micellar Solubilization and Partitioning of TCS

The increased solubility of TCS in the presence of the lauryl sulphate surfactants, together with the observed decrease in antimicrobial activity with increasing surfactant to TCS ratio, support the hypothesis that TCS partitions strongly to the micellar pseudophase of the surfactants. The solubilization behavior inferred from these observations is consistent with published results on the solubilization of structurally similar compounds in ionic surfactant solutions[17]. We have independently confirmed[18] that TCS solubilization in the lauryl sulfate micelles studied here follows a similar model as described in reference 17. The solubilization behavior of TCS in lauryl sulfate micelles can be described according to eq 1;

$$K = \frac{X}{c_w} \tag{1}$$

where K is the micellar binding constant, X is the mole fraction of TCS in the micelle, and c_w is the concentration of TCS in the water (continuous) phase. At TCS concentrations near saturation, K is on the order of 10^2. As the TCS concentration decreases, K behaves according to eq. 2;

$$K = K_0(1 - BX)^2 \tag{2}$$

where K_0 is the value of K as TCS concentration goes to zero, and B>0. B has been interpreted as half the number of surfactant molecules associated with a site for adsorption and is an adjustable parameter of the model. We have estimated K_0 for TCS in lauryl sulfate micelles to be on the order of 10^3. Thus, eq. 2 predicts that K will

increase rapidly as TCS concentration is reduced from a saturated state, suggesting partitioning in favor of the micellar pseudophase as TCS concentration decreases from saturation. Additionally, by eq. 1 we predict that c_w, the concentration of TCS in the water phase (i.e., free or bioavailable) will decrease rapidly as TCS concentration is reduced from a saturated state. Both of these predictions are supported by the antibacterial efficacy results captured in Tables 2 and 3, confirming the applicability of the model for TCS solubilization in lauryl sulfate micelles.

Physicochemical Factors and Antimicrobial Activity of Triclosan

We now see a clear link from the physicochemical properties of TCS in aqueous solution, in solution with added co-solvent, and in surfactant solutions, to its antimicrobial activity in each case. For surfactant solutions, a solubilization model consistent with a large body of experimental evidence on molecules of similar structure and size has been correlated to the observed antibacterial efficacy. Finally, clear evidence supporting a non-specific mode of action of triclosan has been presented. This mode of action should be considered when discussing triclosan resistance in bacterial populations.

REFERENCES

1. Dunaway CS, Christian, SD, and Scamehorn, JF 1995. Overview of the history of solubilization. In Christian SD and Scamehorn JF, editors. Solubilization in surfactant aggregates, New York: Marcel Dekker Inc., pp. 3-21.

2. Bean HS and Berry H 1951. The bactericidal activity of phenols in aqueous solutions of soap. Part II – The bactericidal activity of benzylcholorphenol in aqueous solutions of potassium laurate. J. Pharm. Pharmacol. 3:639-655.

3. Allawala NA and Reigelman S 1953. The release of antimicrobial agents from solutions of surface-active agents. J. Amer. Pharm. Assoc., Sci. Ed. 42(5): 267-274.

4. Allawala NA and Reigelman S 1954. Phenol coefficients and the Ferguson principle. J. Amer. Pharm. Assoc., Sci. Ed. 43(2):93-97.

5. Mitchell AG 1964. Bactericidal activity of chloroxylenol in aqueous solutions of cetomacrogol. J. Pharm. Pharmacol. 16:533-537.

6. Kostenbauder HB 1977. In Block SS, editor. Disinfection, sterilization, and preservation, Philadelphia: Lea & Febiger 2nd Ed., pp. 912-930.

7. Jones RD, Hanuman, BJ, Newman, JL, and Lee, AS 2000. Triclosan: A review of effectiveness and safety in health care settings. Amer. J. Infec. Cont. 28(2): 184-196.

8. Ochs D and Schnyder, M 1997. Concentrated liquid accumulations comprising microbicidally active ingredient. International Patent Application No. WO 97/46218. World Intellectual Property Organization, International Bureau.

9. Moldovanyi L 1996. Surface-active formulations. International Patent Application No. 96/06152. World Intellectual Property Organization, International Bureau.

10. Taylor TJ, Seitz EP, and Fox PS 2000. Compositions comprising a high percent saturation concentration of antibacterial agent. United States Patent No. 6,107,261. United States Patent and Trademark Office.

11. Taylor TJ, Seitz EP, and Fox PS 2000. Compositions comprising a high percent saturation concentration of antibacterial agent. United States Patent No. 6,136,771. United States Patent and Trademark Office.

12. Taylor TJ, Seitz EP, and Fox PS 2002. Compositions comprising a high percent saturation concentration of antibacterial agent. United States Patent No. 6,451,748. United States Patent and Trademark Office.

13. Elworthy PH, Florence AT and Macfarlane CB, 1968. Solubilization by surface-active agents and its applications in chemistry and the biological sciences, London:Chapman and Hall, Ltd., pp. 62-ff.

14. Morrison BM, Scala DD, and Fischler GF, 1997. Topical antimicrobial wash products. In Rieger MM and Rhein LD, editors. Surfactants in cosmetics, 2nd edition, revised and expanded, New York:Marcel Dekker, Inc., pp. 331-356.

15. Regos J and Hitz HR 1974. Investigations on the mode of action of triclosan, a broad spectrum antimicrobial agent. Zbl. Bakt. Hygl, 1.Abt. Orig. A 226: 390-401.

16. McMurry LM, Othinger M, and Levy SB 1998. Triclosan targets lipid synthesis. Nature 394: 531-532.

17. Lee BH, Christian SD, Tucker EE, and Scamehorn JF 1990. Solubilization of mono- and dichlrophenols by hexadecylpyridinium chloride micelles. Effects of substituent groups. Langmuir 6: 230-235.

18. Christian SD and Scamehorn JF, 1998. Final Report: Triclosan interaction with sodium dodecyl sulfate. Surfactant Associates, Inc. under research contract with The Dial Corporation.

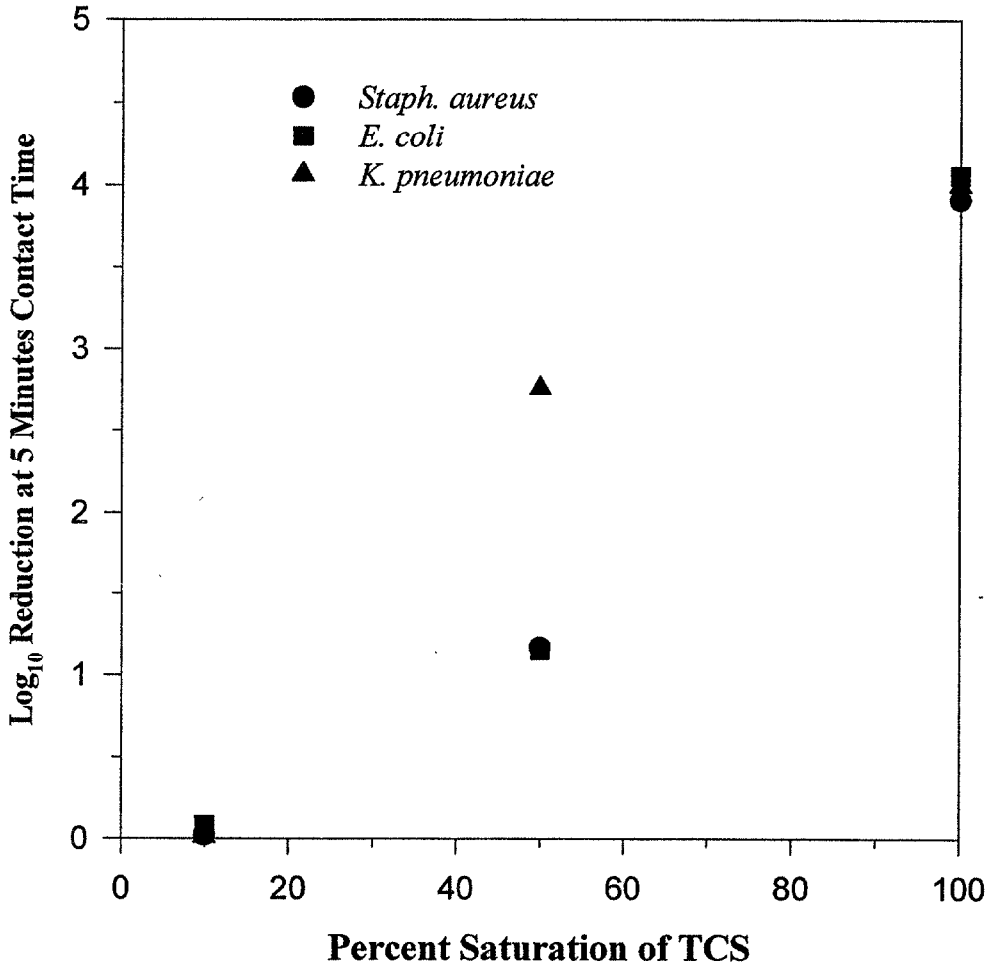

Figure 1 - Log_{10} Reduction vs. Percent Saturation of TCS in Water

TABLE 1 – Effect of Saturation Solubility on Bacterial Kill Kinetics

% TCS	% PG	% Saturation TCS	Time to Effect >4log$_{10}$ Reduction of *S. aureus*
0.0010	0	100	5 minutes
0.0110	24	100	60 seconds
0.1385	50	100	15 seconds

TABLE 2 – Antibacterial Efficacy of TCS in Ammonium Lauryl Sulfate (ALS) Solutions

% TCS	% ALS	% Saturation TCS	Log$_{10}$ Reduction vs. *E. coli* at 1 minute contact time
0	1.35	0	0
0.3	1.35	100	3.95
0.27	1.35	90	2.89
0.21	1.35	70	1.54
0.15	1.35	50	1.17
0.15	0.67	100	3.63

TABLE 3 – Antibacterial Efficacy of TCS as a function of Surfactant:TCS Ratio in Sodium Lauryl Sulfate (SLS)

% TCS	% SLS	% Saturation TCS	Log_{10} Reduction at 5 minutes contact time	
			K. pneum.	*E. coli*
0.3	1.6	100	4.6	4.5
0.3	10	<10	1.16	0.9